Evaluation of glycerol removal techniques, cryoprotectants, and insemination methods for cryopreserving rooster sperm with implications of regeneration of breed or line or both[1,2,3]

P. H. Purdy,*[4] Y. Song,† F. G. Silversides,† and H. D. Blackburn*

*USDA-Agricultural Research Service-National Center for Genetic Resources Preservation, National Animal Germplasm Program, 1111 S. Mason St., Fort Collins, CO 80521-4500; and †Agriculture and Agri-Food Canada, Agassiz Research Centre, PO Box 1000-6947, #7 Highway, Agassiz, British Columbia, Canada, V0M 1A0

ABSTRACT A series of experiments was designed to evaluate the quality of cryopreserved rooster sperm and its fertility so that programs needing to bank germplasm and recreate animals can do so utilizing a minimal amount of cryopreserved semen. In experiment 1, rooster semen from the National Animal Germplasm Program genebank was thawed and glycerol was removed using a discontinuous Accudenz column or by stepwise dilution. The postthaw sperm motilities, plasma membrane integrity, and concentration were determined before and after deglycerolization. Line differences in postthaw sperm concentration and progressive motility were observed before deglycerolization ($P < 0.05$). After glycerol removal, the sperm that was centrifuged through Accudenz had greater total motility (37 vs. 33% sperm; $P < 0.05$), but use of the stepwise dilution method recovered more sperm per milliliter (320.4×10^6) compared with the Accudenz method (239.2×10^6 sperm; $P < 0.05$; range across 6 lines of 165.7 to 581.0 $\times 10^6$ sperm/mL). In experiment 2, rooster semen was cryopreserved using Lake's diluent containing either dimethyl acetamide (DMA) or glycerol as the cryoprotectants. Postthaw analysis revealed that the samples cryopreserved with glycerol survived freezing better, determined by total motility (47.8 and 15.1% glycerol and DMA samples, respectively; $P < 0.05$) and annexin V analyses (1.6 and 11.3% membrane-damaged sperm for glycerol and DMA samples, respectively; $P < 0.05$). Differences in sperm motilities (total and progressive motility) and velocities (path velocity, straight-line velocity, curvilinear velocity) were observed between the 2 cryoprotectant treatments once the glycerol had been removed from those samples cryopreserved with glycerol, of which the glycerol samples had significantly more motile sperm and higher velocities ($P < 0.05$). The fertility of the samples frozen using the 2 cryoprotectants was tested using a single insemination (intravaginal or intramagnal) of 200×10^6 sperm and the fertility (number of live embryos) was evaluated over 18 d. Overall, the intravaginal inseminations had lower fertility than the intramagnal inseminations ($P < 0.05$). In the intravaginal inseminations, the sperm cryopreserved using DMA resulted in lower fertility, but there were no differences in fertility in the intramagnal inseminations due to cryoprotectant ($P > 0.05$). These results indicate that reasonable postthaw sperm quality and fertility can be derived using cryopreserved rooster semen. By utilizing this information, estimations can be made for storing sufficient material for line or breed, or both, recreation programs.

Key words: rooster, spermatozoa, cryopreservation, deglycerolization, artificial insemination

2009 Poultry Science 88:2184–2191
doi:10.3382/ps.2008-00402

INTRODUCTION

Cryopreservation of sperm for artificial insemination is a valuable tool for agricultural species because it allows rapid genetic progress to occur and conservation of valuable genetic resources. In the instance of poultry, however, there are certain problems associated with conserving sperm; only the male genome is

Received September 19, 2008.
Accepted June 18, 2009.
[1]Agassiz Research Centre Publication 760.
[2]Mention of trade name, proprietary products, or specified equipment does not constitute a guarantee by the USDA and does not imply approval to the exclusion of other products that may be suitable. The USDA, Agricultural Research Service, Northern Plains Area, is an equal opportunity-affirmative action employer. All agency services are available without discrimination.
[3]All methods used were approved by the Animal Care Committees of the USDA-Agricultural Research Service Avian Disease and Oncology Laboratory and the Agassiz Research Centre and followed principles described by the Canadian Council of Animal Care for the research performed at these facilities.
[4]Corresponding author: phil.purdy@ars.usda.gov

captured, because roosters are homogametic, and the fertility using a single insemination of frozen-thawed rooster sperm is frequently low (Hammerstedt and Graham, 1992; Buss, 1993; Phillips et al., 1996). Alternatives that are available in other species, such as cryopreservation of oocytes and embryos, are not available currently with poultry. Therefore, because the genetic diversity of agricultural species is shrinking (Blackburn, 2006), it is necessary to preserve the existing genetics using the technology currently available, namely cryopreserved sperm. In doing so, there are many unknown factors specific to poultry sperm cryopreservation that are paramount to programs (genebanks, breed conservation organizations, etc.) concerned with line regeneration, such as the number of inseminations possible from a single insemination straw, differences in survivability of cryopreserved sperm by poultry line, and fertility using cryopreserved samples.

Rooster sperm typically survive cryopreservation very well, as judged by motility evaluated immediately after thawing, but unlike other species, the cryoprotectant typically used (glycerol) must be removed before intravaginal insemination because it is contraceptive (Polge, 1951; Tajima et al., 1989; Hammerstedt and Graham, 1992). A common method of glycerol removal involves stepwise dilution with a glycerol-free medium, centrifugation, and resuspension of the sperm. These methods are time-consuming and decrease the fertility of the sperm due to osmotic and physical damage associated with processing (Hammerstedt and Graham, 1992). Consequently, the sample that survived cryopreservation well has been damaged due to this processing and the fertilizing potential is greatly decreased.

Recently, alternatives to traditional glycerol removal have been investigated by Long and Kulkarni (2004), who reported a method for removing glycerol from fresh rooster sperm and cryopreserved rooster sperm by centrifugation through a discontinuous Accudenz column (Accurate Chemical & Scientific Corp., Westbury, NY) without the stepwise dilution process. An alternative cryoprotectant, dimethyl acetamide (**DMA**), has also been investigated as a means of circumventing the use of glycerol for cryopreservation of rooster sperm (Bacon et al., 1986; Chalah et al., 1999; Woelders et al., 2006; Blesbois et al., 2007). The reported benefit of using DMA is that it is not contraceptive and therefore does not require removal from frozen-thawed sperm. Thus, damage to the sperm normally caused by glycerol removal before insemination is minimized and therefore may result in greater fertility.

The purpose of this study was to 1) evaluate methods for removing glycerol from frozen-thawed sperm, 2) compare the postthaw quality of rooster sperm that was cryopreserved using glycerol or DMA, and 3) compare the fertility of frozen-thawed rooster sperm that was cryopreserved with either glycerol or DMA and inseminated either intravaginally or intramagnally. The cryoprotectants, DMA and glycerol, were selected for analysis because these are those most commonly used by other genebanks such as the Netherlands (Woelders et al., 2006) and France (Blesbois et al., 2007). The data from these experiments will be used to determine quantities of samples required for line regeneration for national conservation programs.

MATERIALS AND METHODS

Experimental Design

Experiment 1 was performed to compare Accudenz or stepwise dilution methods of glycerol removal from frozen-thawed rooster sperm. Samples for this analysis were from 6 different lines as described in Table 1 and were frozen individually (not pooled) in this experiment. Immediately after thawing, 2 straws per rooster ejaculate were analyzed separately for sperm motility, concentration, and plasma membrane integrity. Aliquots from each sample were processed to remove the glycerol from the rooster sperm by either Accudenz centrifugation or stepwise dilution and centrifugation. After glycerol removal, the resulting samples were analyzed to determine the volume of recovered sperm, sperm concentration, motility, and plasma membrane integrity. Semen straws were processed separately to replicate repopulation conditions.

Experiment 2 compared the postthaw quality and fertility of rooster sperm when glycerol or DMA were used as cryoprotectants and intramagnal and intravaginal insemination was used for 2 breeds, Rhode Island Red and White Leghorn. The control treatment for each breed was a group of birds that was inseminated using fresh semen via intravaginal insemination. Rooster semen samples from the Rhode Island Red line 50 (n = 20) and White Leghorn blue line (Table 1; n = 26) roosters were collected and semen was pooled within each breed. The pooled samples were split and cryopreserved in diluents containing 1 of the 2 cryoprotectants. The postthaw quality [sperm motility, plasma membrane integrity, and plasma membrane phosphatidylserine (**PS**) exposure] was then evaluated before and after glycerol removal or immediately after thawing for the DMA samples using 4 semen straws from each of the breed and cryoprotectant combinations. Glycerol removal was performed using the stepwise dilution method used in experiment 1.

Semen Collection, Processing, Cryopreservation, and Thawing

The samples were collected from roosters by abdominal massage (Burrows and Quinn, 1937). After collection, samples that were to be frozen using glycerol as the cryoprotectant were diluted 1:1 (vol/vol) with glycerol-free Lake's diluent (Lake and Stewart, 1978), which was precooled to 5°C. The samples were then placed in a rack on ice and transported to the laboratory (<15 min). Samples were diluted 1:2 (vol/vol; sample to cryopreservation medium) with Lake's diluent

Table 1. Description of the rooster lines acquired from the USDA Avian Disease and Oncology Laboratory (ADOL) and from the Agriculture and Agri-Food Canada Agassiz Research Centre used in this study

Description[1]
1) Cornell University line P; selected for tumor development after inoculation with the Jozef Marek (JM) strain of Marek's disease virus (n = 4). Used in experiment 1.
2) Reaseheath line C; developed by full-sibling matings; selection for large family size; utilized for research on avian leukosis viruses and immune response traits (n = 4). Used in experiment 1.
3) ADOL line 15B1; closed line; 95% inbred; selected for susceptibility to avian leukosis virus (n = 4). Used in experiment 1.
4) ADOL line O; closed line; selected for absence of endogenous avian leukosis virus proviral genesgenes and for susceptibility to avian leukosis virus (n = 4). Used in experiment 1.
5) ADOL congenic line 15.15I_5; closed line; 99% inbred; selected for susceptibility to lymphoid leucosis and Marek's disease (n = 4). Used in experiment 1.
6) ADOL recombinant congenic line 6C.7W; selected for tumor resistance or susceptibility (n = 3). Used in experiment 1.
7) Rhode Island Red line 50 (n = 20). Used in experiment 2.
8) White Leghorn blue line (n = 26). Used in experiment 2.

[1]Lines 1 through 6 are described in Bacon et al. (2000), and lines 7 and 8 are described in Silversides et al. (2007).

containing glycerol (11% final concentration, 5°C) in a 5°C room and loaded into 0.5-mL CBS straws (IMV, Minneapolis, MN).

Samples frozen using DMA (experiment 2) were collected and diluted with Lake's diluent immediately after collection as described previously. Samples were then diluted 1:2 (vol/vol) with Lake's diluent containing DMA (6% final concentration; Blesbois et al., 2007) and loaded into 0.5-mL CBS straws.

Samples diluted with glycerol cryoprotectant were frozen in an extruded polystyrene foam box containing liquid nitrogen vapor (6.4 cm above liquid nitrogen) for 10 min (10°C/min) and plunged into the liquid nitrogen for storage (Phillips et al., 1996). Samples containing DMA were placed on a rack, frozen in liquid nitrogen vapor (1 cm above liquid for 7 min), and plunged into the liquid for storage, which resulted in a 59°C/min freeze rate, an attempt to approximate the 50°C/min rate reported by Blesbois et al. (2007).

Immediately before analysis, samples cryopreserved using glycerol were thawed in a 5°C water bath for 5 min (Phillips et al., 1996). The samples frozen using DMA were thawed in a 50°C water bath for 20 s (Blesbois et al., 2007).

Evaluation of Sperm Motility and Concentration

Sperm motility was determined using a computer-assisted sperm analyzer (Hamilton Thorne Motility Analyzer; IVOS, Beverly, MA). An aliquot of each sample (5 μL) was diluted with 100 μL of Lake's diluent containing glycerol for postthaw analysis or with glycerol-free Lake's diluent for postglycerol removal analysis. A subsample of this solution (5 μL) was placed on a Standard Count Analysis Chamber (Spectrum Technologies, Healdsburg, CA) and analyzed for motility. The following settings were used for computer-assisted sperm analyzer analysis: 30 frames acquired, frame rate of 60 Hz, minimum contrast of 25, minimum cell size of 4 pixels, path velocity (**VAP**) cutoff of 5 μm/s, progressive minimum VAP cutoff of 50 μm/s, progressive velocity cutoff of 10 μm/s, static head size of 0.27 to 4.24, magnification of 1.95, and a minimum of 500 sperm from at least 5 fields were observed for motility analysis.

Samples from each straw were analyzed for sperm concentration using a SpermaCue spectrophotometer (Minitube, Verona, WI) that was calibrated for rooster sperm. Samples were diluted 1:1 or 1:2 (vol/vol) with Lake's diluent and the optical density (sperm concentration) was determined.

Glycerol Removal

Solutions of Accudenz, 30 and 12%, were prepared according to McLean et al. (1998) and maintained at 5°C. Discontinuous columns of Accudenz were prepared in 1.5-mL flat-top microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) by layering 1 mL of 12% Accudenz over 0.1 mL of 30% Accudenz. An aliquot from each sample (150 μL) was placed on top of the column and centrifuged at 300 × *g* for 25 min in a 5°C room. The volumes of Accudenz and the frozen-thawed semen are one-fifth of the volumes used by others (McLean et al., 1998; Long and Kulkarni, 2004; Peláez and Long, 2007), but initial evaluation of decreasing the centrifugation speed to 250 × *g* (one-fifth of the speed reported by the same authors) left a significant amount of sperm in the 12% Accudenz fraction (P. H. Purdy, unpublished data). Consequently, the speed was adjusted to 300 × *g*, which resulted in the sperm being present at the interface between the 12 and 30% layers after centrifugation (P. H. Purdy, unpublished data). The 12 and 30% Accudenz layers were then removed according to McLean et al. (1998) and the resulting quantity of sperm was measured to determine volume and sperm concentration, as described previously.

The stepwise dilution method was carried out in a 5°C room using glycerol-free Lake's diluent at 5°C. The diluent was added every minute in the following increments: 10 × 10 μL (10 dilutions of 10 μL per dilution), 10 × 20 μL, 10 × 50 μL, and 5 × 100 μL (Tajima et al., 1989; Phillips et al., 1996) and then centrifuged 300 × *g* for 25 min. After centrifugation, the supernatant was discarded and the pellets were measured to determine

volume and sperm concentration, as described previously.

Flow Cytometry

Plasma membrane integrity (Donoghue et al., 1995) was determined via flow cytometry using a minimum of 5,000 sperm per treatment sample. Lake's diluent, with glycerol for immediate postthaw and glycerol-free for postglycerol removal analysis, was used to dilute 5-μL samples of semen. The fluorescent stains SYBR-14 (Molecular Probes, Eugene, OR; 5 μL of a 20-μ*M* solution in dimethyl sulfoxide) and propidium iodide (**PI**; 10 μL of a 2.4 m*M* solution in water) were added to the diluted semen (10×10^6 sperm/mL) and incubated at 23°C for 10 min in the dark. The samples were analyzed using a CyAN ADP flow cytometer (DakoCytomation, Fort Collins, CO) equipped with an argon laser (488 nm) at 20 mW of power, the FL-1 detector (530-nm band pass filter) to detect SYBR-14 positive-PI-negative sperm (plasma membrane intact), and the FL3 detector (613-nm band pass filter) to detect PI-positive-SYBR-14-negative (plasma membrane compromised) sperm.

Phosphatidylserine is normally present on the inner leaflet of the plasma membrane, but when sperm are altered due to such things as capacitation, the acrosome reaction, or cryopreservation damage, PS will be exposed (Glander and Schaller, 1999). Phosphatidylserine exposure was determined using the staining combination of Annexin V (Molecular Probes; PS exposure) and plasma membrane integrity (PI). Semen samples were thawed as described previously and maintained at 41°C. Aliquots (1×10^6 sperm per sample) were diluted into 100 μL of annexin-binding buffer (10 m*M* HEPES, 140 m*M* NaCl, 2.5 m*M* $CaCl_2$, pH 7.4) containing 5 μL of Annexin V and 5 μL of PI according to the instructions of the manufacturer. The samples were incubated with the stains for 15 min at 23°C, diluted with 900 μL of annexin-binding buffer, and analyzed immediately after thawing for both DMA and glycerol samples and after glycerol removal (glycerol samples only). The flow cytometer was set up in the same manner as for the SYBR-14-PI analyses, but in this instance, the 530-nm band pass filter will detect PS-positive sperm. The autocompensation feature of the flow cytometer was used to assist in separation of the sperm populations.

Insemination and Fertility

One hundred fifty-seven White Leghorn and Rhode Island Red hens, aged 52 wk, were randomly assigned to a cryoprotectant protocol and insemination method. The hens were housed in cages in groups of 2 to 3 birds per cage, by breed, and all birds in a cage were inseminated only once with the same treatment. Intravaginal inseminations were performed using 200×10^6 sperm of fresh pooled sperm, or frozen-thawed sperm from either the DMA samples or the glycerol samples. The DMA samples were thawed and inseminated without further processing, whereas the glycerol samples were thawed and processed to remove the cryoprotectant. No postthaw processing was performed for the intramagnal inseminations. All samples were inseminated within 10 min of the completion of glycerol removal or thawing in the case of DMA samples and intramagnal glycerol samples.

Intramagnal insemination was performed as described by Song and Silversides (2007). The hens were anaesthetized by injection of 0.15 mL (White Leghorn) or 0.20 mL (Rhode Island Red) of xylazine (20 mg/mL) into the brachial vein. The left leg of the hen was drawn up while the hen was lying on its right side. Feathers overlying the left abdominal wall were removed and the area around the site of the surgery was wetted with 70% ethanol. An incision of approximately 2.5 cm was made in the skin between the thigh and the breast close to the last rib and an incision of approximately 1 cm was made in the underlying muscle where it is thinnest. A retractor was used to expose the magnum, which lies alongside the body wall in this area of the abdomen. A section of the magnum was held with large forceps while the sperm suspension (200×10^6 sperm) was injected using a 1-mL syringe equipped with a 20-gauge needle. The exposed loop of the magnum was then returned to the peritoneal cavity and the skin was closed by continuous sutures.

Eggs were collected daily, stored at 18°C, and set twice a week. Fertility was determined after 6 d of incubation by breaking the eggs. Live embryos were clearly distinguishable from dead embryos and other material of embryonic origin (blood and membranes). Eggs were considered fertile but dead early in incubation if blood or membranes were present.

Statistics

In experiment 1, differences in sperm motilities, concentration, and plasma membrane integrity before and after glycerol removal were analyzed using a mixed model ANOVA (PROC MIXED ANOVA) and type 3 sums of squares (SAS Institute, 1985). The model included as fixed effects glycerol removal method, and line; random effects included rooster within line, and semen straw within rooster and within line.

In experiment 2, differences in postthaw motilities, plasma membrane integrity, and the percentage of sperm displaying phosphatidyl serine were analyzed using a mixed model ANOVA (PROC MIXED ANOVA) for the main effects of rooster, breed (fixed), cryoprotectant (fixed), and straw (random), which was replicated (random effect within line; SAS Institute, 1985). The fertility measures were analyzed using a GLM model that consisted of breed, treatment, and breed × treatment interaction, all of which were fixed effects. Cage was the experimental unit for this portion of the experiment. The experiment was an incomplete factorial; therefore, 5 treatments were formed as: 1) control

Table 2. Characteristics of frozen-thawed rooster sperm samples, by line, before glycerol removal[1]

Line (no.)[2]	Concentration (10^6)	Motility (%)	Progressive motility (%)	PMI[3] (%)
1 (4)	165.7[b]	50	28[ab]	56
2 (4)	482.7[a]	37	10[c]	56
3 (4)	502.9[a]	32	13[c]	36
4 (4)	456.9[a]	40	21[abc]	39
5 (4)	482.4[a]	43	18[bc]	38
6 (3)	581.0[a]	57	32[a]	49
SEM	25.9	2.1	1.3	3.2

[a–c]Means within a column lacking a common superscript differ ($P < 0.05$).

[1]Two straws per rooster ejaculate were analyzed.

[2]Individual lines are described in Table 1, and the number in parentheses indicates the number of males used from that line.

[3]Plasma membrane integrity.

(fresh semen inseminated intravaginally), 2) DMA and intramagnal insemination, 3) glycerol and intramagnal insemination, 4) DMA and intravaginal insemination, and 5) glycerol and intravaginal insemination. Each cage had multiple hens; therefore, actual fertility measurements were constructed as fertility per egg laid per cage per day, fertility per egg per hen per day, and total fertile eggs per hen.

RESULTS

Experiment 1: Accudenz and Stepwise Dilution Analyses

Significant line differences in sperm concentration and progressive motility before glycerol removal were observed (Table 2). From the results presented in Table 2, the number of insemination doses per straw was also determined by line. If 100×10^6 total sperm per dose are to be inseminated (Long and Kulkarni, 2004), then from the lines analyzed, 0.84 to 2.91 inseminations can be performed from a single 0.5-mL straw, whereas more conservative methods of insemination (200×10^6 sperm; Phillips et al., 1996) would result in half of the insemination doses.

The method of glycerol removal affected the resulting sperm concentration. Use of the dilution method recovered significantly more sperm that had more intact plasma membranes than the Accudenz method ($P < 0.05$; Table 3). The difference in postglycerol removal sperm concentration (68.7×10^6 sperm) results in a significant loss (0.68 to 0.34 doses based on a 100 or 200 $\times 10^6$ sperm insemination dose, respectively) of insemination doses due to processing with the Accudenz centrifugation method. The Accudenz method did produce samples with significantly greater motility, increased elongation, and increased straightness compared with the dilution method, but the dilution method produced samples with greater plasma membrane integrity (Table 3).

Experiment 2: Cryoprotectant Comparison

Postthaw semen characteristics for glycerol and DMA comparison are presented in Table 4. No significant breed differences in postthaw sperm quality were observed for Rhode Island Red and White Leghorn roosters; therefore, only the differences in cryoprotectant are reported. Samples cryopreserved using glycerol had greater motility and quality of motion (VAP, progres-

Table 3. Means of the motility characteristics of frozen-thawed rooster sperm samples after dilution and centrifugation (Dilution) or centrifugation through a discontinuous Accudenz column[1] (Accudenz) for removal of glycerol (n = 23 roosters; 2 semen straws per rooster)

Sperm attribute[2]	Accudenz	Dilution	SEM	Significance
Concentration (10^6 sperm/mL)	239.2[a]	320.4[b]	17.2	0.02
Total motility (%)	37[a]	33[b]	1.0	0.02
Progressive motility (%)	13	12	0.6	0.05
Plasma membrane integrity (%)	55[a]	57[b]	0.6	0.04
Elongation (%)	30.8[a]	29.9[b]	0.2	0.04
Area (μm^2)	6.8	6.5	0.06	0.3
Path velocity (VAP, μm/s)	39.6	38.3	0.7	0.4
Progressive velocity (VSL; μm/s)	31.7	29.7	0.6	0.3
Track speed (VCL; μm/s)	76.3	71.5	1.0	0.2
Straightness (ratio of VSL:VAP)	68.5[a]	64.7[b]	0.3	0.03
Linearity (ratio of VSL:VCL)	35.3	33.8	0.4	0.3
Lateral head amplitude (μm)	3.4	3.3	0.04	0.7
Beat cross frequency (frequency/s)	35.0	33.9	0.3	0.9

[a,b]Means within a row lacking a common superscript differ ($P < 0.05$).

[1]Accurate Chemical & Scientific Corp., Westbury, NY.

[2]VAP = path velocity; VSL = straight-line velocity; VCL = curvilinear velocity.

Table 4. Postthaw results of motility and flow cytometric analysis of rooster sperm from 2 breeds (Rhode Island Red and White Leghorn) of poultry cryopreserved using the cryoprotectants (CPA; n = 3 straws per CPA treatment) dimethyl acetamide (DMA) or glycerol (GLY+) and analyzed immediately after thawing or after thawing, stepwise dilution, and centrifugation to remove glycerol (GLY−)[1]

CPA[2]	DMA	GLY+	GLY−	SEM
Concentration (10^6 sperm/mL)	804[a]	808[a]	529[b]	28.1
Motility (%)	15.1[c]	47.8[a]	28[b]	1.4
Progressive motility (%)	1.8[c]	20.3[a]	8.6[b]	0.95
VAP	36.8[c]	61.0[a]	52.9[b]	1.3
VSL	26.8[c]	50.0[a]	42.3[b]	1.2
VCL	78.9[c]	108.9[a]	96.3[b]	2.1
ALH	5.7[a]	4.9[b]	4.8[b]	0.8
BCF	27.9[b]	30.8[a]	31.2[a]	0.5
Straightness	74[b]	77.9[a]	75.4[b]	0.4
Linearity	38.3[c]	44.9[a]	42.5[b]	0.4
Elongation	32.4[b]	34.4[a]	33.3[ab]	0.3
Area	6.9[b]	7.9[a]	7.3[b]	0.2
PS positive (%)	11.3[a]	1.6[b]	14.7[a]	0.6
PMI (%)	59.7	59.5	56.6	1.9

[a–c]Means within a row lacking a common superscript differ ($P < 0.05$).

[1]Statistical analyses were performed on the data combined from the 2 breeds because no statistical differences were observed due to breed.

[2]VAP = path velocity; VSL = straight-line velocity; VCL = curvilinear velocity; ALH = lateral head amplitude; BCF = beat cross frequency; PS = phosphatidylserine; PMI = plasma membrane intact.

sive velocity, curvilinear velocity) as well as greater membrane quality, as indicated by lower PS exposure, immediately after thawing compared with the DMA treatment (Table 4). Glycerol removal by stepwise dilution (Table 4) resulted in significant alterations of the motility, quality of motion characteristics, and plasma membrane PS exposure compared with the same samples before undergoing the process of glycerol removal. However, the samples that underwent glycerol removal still had more robust motion metrics than the samples cryopreserved using DMA (Table 4). Sperm treated by all 3 approaches had similar percentages of intact plasma membranes ($P > 0.05$). However, the cell area was significantly larger for those cells cryopreserved in glycerol than the DMA and the glycerol + stepwise dilution.

Fertility of Cryopreserved Treatments

An ANOVA using GLM was performed in which semen treatment, breed, and the semen treatment × breed interaction were included in the model. The model yielded consistent results for all response variables analyzed. Semen treatment effect was found to be significant for all measures of fertility, whereas breed and treatment × breed were found to be nonsignificant sources of variation. Linear contrasts were performed to compare the 2 methods of insemination and the 2 cryoprotectants for the response variables. The intramagnal method had significantly higher fertility levels then the intravaginal approach (Table 5). The results also indicate no significant difference between the types of cryoprotectant used for all measures of fertility (Table 5). Confounding does exist because samples cryopreserved in glycerol were handled differently (the glycerol + intravaginal insemination samples underwent glycerol removal via the stepwise dilution method).

DISCUSSION

Achieving fertility using frozen-thawed rooster sperm relies on many factors related to sperm quality, postthaw processing, and method of insemination. Experiment 1 was an evaluation of postthaw glycerol removal methods involving multiple poultry lines. The line differences in postthaw motility observed in this research (experiment 1) are similar to those reported elsewhere for motility (Yousif et al., 1984) and mobility (Froman and Kirby, 2005). The stepwise dilution method will successfully remove glycerol from rooster sperm but it requires attention to detail during the multiple dilutions and is more time-consuming (60 min total) compared with the Accudenz method (30 min total), although the latter requires preparation of the column and layering of the sample before centrifugation. Potentially, the number of dilutions in the stepwise method could be decreased, which would minimize the total processing time (Lake et al., 1981).

The Accudenz method yielded greater postprocessing motility, elongation, and straightness of movement, but the stepwise dilution method resulted in 34% more sperm, with more intact plasma membranes, available for insemination when compared with the Accudenz methodology. In trying to maximize the use of stored semen, the additional number of sperm available for insemination with the stepwise approach compared with the Accudenz method is important. The additional number of motile sperm available with the stepwise method may be more important than an increase in the percentage of motile cells obtained with the Accudenz

Table 5. Least squares means for total fertile eggs laid per cage, fertility per egg per cage per day, fertility per egg laid per hen per day, and total fertile eggs per hen from d 2 to 8 and 2 to 18 after insemination by treatment

			Days 2 to 8 after insemination			Days 2 to 18 after insemination		
Semen-insemination treatment[1]	Number of cages used	Hens	Total fertile eggs per cage (SEM)	Fertility per egg laid per day per cage (SEM)	Fertility per egg laid per hen per day (SEM)	Total fertile eggs per cage (SEM)	Fertility per egg laid per day per cage (SEM)	Fertility per egg laid per hen per day (SEM)
Control	12	31	10.8 (0.77)	0.83 (0.08)	0.36 (0.08)	18.2 (1.48)	0.60 (0.06)	0.25 (0.03)
Glycerol IV[b]	15	35	2.2 (0.69)	0.23 (0.07)	0.14 (0.07)	2.4 (1.33)	0.09 (0.05)	0.05 (0.03)
Glycerol IM[a]	12	29	2.5 (0.77)	0.49 (0.09)	0.23 (0.09)	4.3 (1.48)	0.30 (0.06)	0.13 (0.03)
DMA IV[b]	13	32	0.7 (0.74)	0.13 (0.08)	0.07 (0.08)	1.1 (1.43)	0.10 (0.06)	0.05 (0.03)
DMA IM[a]	12	30	3.6 (0.77)	0.57 (0.08)	0.25 (0.08)	7.7 (1.48)	0.41 (0.06)	0.18 (0.03)

[a,b]Linear contrasts between methods of artificial insemination were significant for all traits analyzed within the 2 to 8- and 2 to 18-d period ($P < 0.05$).

[1]Control semen-insemination treatments represent fresh semen and an intravaginal insemination method. Cryoprotectants [dimethylacetamide (DMA), glycerol] were not significantly different. Use of the glycerol cryoprotectant required postthaw stepwise dilution to remove glycerol for the intravaginal inseminations (IV) but not for the intramagnal (IM) inseminations, whereas the DMA samples did not require removal of the cryoprotectant before insemination with either method.

method. This result underscores the need to better understand the relationship between motility and fertility levels. Because maximization of stored semen is a concern for genebanks, therefore, at present, the stepwise dilution method would better meet reconstitution purposes and therefore it was used it in experiment 2. Still, because no fertility trials comparing frozen-thawed rooster sperm, which have had the glycerol removed by either the Accudenz or stepwise dilution method, have been performed, it may be prudent to perform in the future.

The range of motility values observed in experiment 2 was in agreement with other studies that used glycerol as the cryoprotectant, as was the range of plasma membrane integrity values (Seigneurin and Blesbois, 1995; Phillips et al., 1996). Furthermore, the significant differences between the samples cryopreserved with glycerol (before and after glycerol removal) and DMA have also been documented previously (Chalah et al., 1999). A potential cause for the low levels of motility with the DMA samples could be attributed to the freeze rate. In this experiment, the samples were frozen using the available method, which was a box and static liquid nitrogen vapor resulting in a 59°C/min freeze rate, which was similar to the 50°C rate reported by Blesbois et al. (2007). If the freeze rate was increased to 200°C/min, as demonstrated by Woelders et al. (2006), potentially there could have been an increase in postthaw motility and quality. Unfortunately, access to a programmable freezer to create a 200°C/min rate was not available at the time but will be explored in the future.

The decrease in motility and changes in sperm quality (PS exposure) before and after glycerol removal (stepwise dilution method; experiment 2) attest to the fragile nature of frozen-thawed rooster sperm. These results underscore the care with which frozen-thawed rooster sperm must be treated through the dilution and centrifugation process to minimize cellular damage. In addition, it has been our experience that if strict attention to handling is not adhered to, the use of frozen-thawed sperm is greatly compromised. Although there were no differences in plasma membrane integrity, a more subtle indicator of sperm quality, PS exposure, which is indicative of poor plasma membrane quality (Glander and Schaller, 1999), increased due to the glycerol removal process. Although no differences in PS exposure in the samples at the time of insemination were observed, these analyses once again demonstrate the damaging effects of the glycerol removal process. Due to the sensitivity of PS, this test may have a role to play as an alternative method for evaluating rooster sperm, a method that couples plasma membrane integrity analysis with a membrane quality analysis (Annexin V; Glander and Schaller, 1999).

Examination of the experiment 2 fertilizing potential demonstrated that sperm samples cryopreserved in glycerol and before undergoing stepwise dilution generally had significantly better motility and cellular performance characteristics than did the DMA and glycerol stepwise dilution treatments (Table 4). These measures did not translate into an advantage for fertility measures, in which the DMA intramagnal inseminations resulted in higher fertility levels (Table 5). This suggests that the intramagnal insemination is perhaps more robust and semen with a wider range of quality can be used successfully. This is particularly evident in comparing the fertility results of the intravaginal and intramagnal inseminations, in which DMA was used as the cryoprotectant.

The results indicated that intramagnal insemination yielded the highest fertility levels regardless of cryoprotectant used, which is in agreement with results reported by Bacon et al. (1986). The intravaginal inseminations resulted in fertility that was within the range of other reports (Phillips et al., 1996; Blesbois et al., 2007), but it must be emphasized that in this experiment, hens were only inseminated once over the evaluation period of 18 d. Other research using cryopreserved rooster sperm resulted in fertility rates consistently greater than 60%, but this involved inseminations every

3 to 4 d using 300×10^6 sperm per insemination (Chalah et al., 1999; Tselutin et al., 1999; Woelders et al., 2006), all of which are methodologies that attempted to maximize fertility using multiple inseminations of moderate sperm numbers (Brillard, 1993). The current research only used 200×10^6 sperm per insemination (Buss, 1993; Phillips et al., 1996) and single inseminations to maximize the number of doses attainable from cryopreserved samples and to determine the number of fertile eggs attainable from single inseminations. By increasing the number of inseminations performed, the fertility would increase.

This research was performed to replicate line-breed regeneration that may occur with national conservation programs and therefore, only single inseminations were performed because limited cryopreserved samples would be available from these programs. Because we endeavored to replicate regeneration conditions, we chose to evaluate both intravaginal and intramagnal inseminations. It is apparent that although both of these techniques have limitations, the intramagnal technique is invasive and may not be readily usable by many institutions. Still, in the event that drastic measures need to be employed for purposes of line-breed regeneration, utilizing intramagnal inseminations may be the best option to ensure greater levels of fertility with fewer frozen-thawed samples. Furthermore, the lack of significant breed differences for fertility suggests that the intramagnal insemination is a robust approach.

From this set of experiments, several conclusions relevant to genebanking poultry semen can be derived. First, intramagnal insemination appears more robust, suggesting that it be the insemination method of choice for regeneration-expansion of lost or endangered lines. Given the insemination method, type of cryoprotectant used becomes less important. However, utilization of DMA does have a slight but nonsignificant advantage over glycerol. Finally, these results suggest that breed effect may be a nonsignificant source of variation when intramagnal insemination is employed, but this would need to be further investigated.

REFERENCES

Bacon, L. D., H. D. Hunt, and H. H. Cheng. 2000. A review of the development of chicken lines to resolve genes determining resistance to diseases. Poult. Sci. 79:1082–1093.

Bacon, L. D., D. W. Salter, J. V. Motta, and L. B. Crittenden. 1986. Cryopreservation of chicken semen of inbred or specialized strains. Poult. Sci. 65:1965–1971.

Blackburn, H. D. 2006. The National Animal Germplasm Program: Challenges and opportunities for poultry genetic resources. Poult. Sci. 85:210–215.

Blesbois, E., F. Seigneurin, I. Grasseau, C. Limouzin, J. Besnard, D. Gourichon, G. Coquerelle, P. Rault, and M. Tixier-Boichard. 2007. Semen cryopreservation for ex situ management of genetic diversity in chicken: Creation of the French avian cryobank. Poult. Sci. 86:555–564.

Brillard, J. P. 1993. Sperm storage and transport following natural mating and artificial insemination. Poult. Sci. 72:923–928.

Burrows, W. H., and J. P. Quinn. 1937. Collection of spermatozoa from the domestic fowl and turkey. Poult. Sci. 16:19–24.

Buss, E. G. 1993. Cryopreservation of rooster sperm. Poult. Sci. 72:944–954.

Chalah, T., F. Seigneurin, E. Blesbois, and J. P. Brillard. 1999. In vitro comparison of fowl sperm viability in ejaculates frozen by three different techniques and relationship with subsequent fertility in vivo. Cryobiology 39:185–191.

Donoghue, A. M., D. L. Garner, D. J. Donoghue, and L. A. Johnson. 1995. Viability assessment of turkey sperm using fluorescent staining and flow cytometry. Poult. Sci. 74:1191–1200.

Froman, D. P., and J. D. Kirby. 2005. Sperm mobility: Phenotype in roosters (*Gallus domesticus*) determined by mitochondrial function. Biol. Reprod. 72:562–567.

Glander, H. J., and J. Schaller. 1999. Bionding of annexin V to plasma membranes of human spermatozoa: A rapid assay for detection of membrane changes after cryostorage. Mol. Hum. Reprod. 5:109–115.

Hammerstedt, R. H., and J. K. Graham. 1992. Cryopreservation of poultry sperm: The enigma of glycerol. Cryobiology 29:26–38.

Lake, P. E., O. Ravie, and J. McAdam. 1981. Preservation of fowl semen in liquid nitrogen: Application to breeding programmes. Br. Poult. Sci. 22:71–77.

Lake, P. E., and J. M. Stewart. 1978. Preservation of fowl semen in liquid nitrogen–An improved method. Br. Poult. Sci. 19:187–194.

Long, J. A., and G. Kulkarni. 2004. An effective method for improving the fertility of glycerol-exposed poultry semen. Poult. Sci. 83:1594–1601.

McLean, D. J., A. J. Feltmann, and D. P. Froman. 1998. Transfer of sperm into a chemically defined environment by centrifugation through 12% (wt/vol) Accudenz®. Poult. Sci. 77:163–168.

Peláez, J., and J. A. Long. 2007. Characterizing the glycocalyx of poultry spermatozoa: I. Identification and distribution of carbohydrate residues using flow cytometry and epifluorescence microscopy. J. Androl. 28:342–352.

Phillips, J. J., R. K. Bramwell, and J. K. Graham. 1996. Cryopreservation of rooster sperm using methyl cellulose. Poult. Sci. 75:915–923.

Polge, C. 1951. Functional survival of fowl spermatozoa after freezing at −79°C. Nature 167:949–950.

SAS Institute. 1985. User's Guide: Statistics. Version 5 edition. SAS Institute Inc., Cary, NC.

Seigneurin, F., and E. Blesbois. 1995. Effects of the freezing rate on viability and fertility of frozen-thawed fowl spermatozoa. Theriogenology 43:1351-1358.

Silversides, F. G., D. M. Shaver, and Y. Song. 2007. Pure line laying chickens at the Agassiz Research Centre. Animal Genetic Resources Information 40. FAO, Rome, Italy.

Song, Y., and F. G. Silversides. 2007. Heterotopic transplantation of testes in newly hatched chicken and production of viable offspring via intramagnal insemination. Biol. Reprod. 76:598–603.

Tajima, A., E. F. Graham, and D. M. Hawkins. 1989. Estimation of the relative fertilizing ability of frozen chicken spermatozoa using a heterospermic competition method. J. Reprod. Fertil. 85:1–5.

Tselutin, K., F. Seigneurin, and E. Blesbois. 1999. Comparison of cryoprotectants and methods of cryopreservation of fowl spermatozoa. Poult. Sci. 78:586–590.

Woelders, H., C. A. Zuidberg, and S. J. Hiemstra. 2006. Animal genetic resources conservation in the Netherlands and Europe: Poultry perspective. Poult. Sci. 85:216–222.

Yousif, Y. F., G. A. Ansah, and R. B. Buckland. 1984. Effect of selection for fertility of frozen-thawed semen in chickens on the fertility of fresh and stored semen. Poult. Sci. 63:1475–1480.